The molecular role of Serf2 in development and misfolded protein aggregation

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Chapter 4

Investigation into the Sox1-Cre mice as a model for brain specific gene deletion

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Abstract

The Cre-*loxP* system is a widely used strategy to cause gene deletions in specific tissues or cells in order to avoid lethal effects of whole body gene deletion. However, it is known that use of Cre mouse lines can have adverse side effects, which has not yet been investigated for most Cre lines. Here, we investigated the effect of the Sox1-Cre mouse line that is used for gene deletion throughout the nervous system. We observed a slightly reduced brain weight and cortical surface, however no other obvious brain defects could be detected in these mice. Future studies using the Sox1-Cre mouse model should include the correct controls to avoid possible effects caused by the Sox1-Cre mouse line.

Introduction

Homologous recombination is an often used gene targeting technology to generate knockout mice, in order to study gene function. This method is limited when loss of the gene of interest results in a lethal phenotype. In 1981 the Cre-*loxP* system was introduced. Cre recombinase is an enzyme from bacteriophage P1, that specifically catalyzes recombination between two 34-bp *loxP* recognition sites (Abremski and Hoess, 1984). The Cre-*loxP* technology makes it possible to delete a gene in a specific organ or cell type, by inserting *loxP* recognition sites around the gene of interest and expressing Cre under a tissue or cell type specific promoter. Several mouse models have been generated that express Cre under different promoters to target the whole brain, a specific brain region or a subset of brain cells (Gavériaux-Ruff and Kieffer, 2007).

In order to express Cre throughout the whole brain only a few promoters are used, such as the Nestin (Tronche et al., 1999) and Sox1 (Takashima et al., 2007) promoter. The Nestin-Cre mouse strain is with over 800 citations, the most commonly used model to generate a gene deletion in the entire nervous system. It has however been reported that Nestin is also expressed in non-neuronal tissues, like the kidney and pancreas (Delacour et al., 2004; Dubois et al., 2006). Moreover, Nestin-Cre animals have significantly decreased body weight (Declercq et al., 2015; Giusti et al., 2014) and show reduced performance in the fear conditioning test (Giusti et al., 2014). Due to these limitations, the Nestin-Cre mouse model is not an ideal model for studying the function of genes in the brain. Another option for whole brain gene targeting is the more recently developed Sox1-Cre mouse line (Takashima et al., 2007). Sox1 is a transcription factor involved in neuronal induction and has an exclusive expression in neuronal precursors throughout development and is down regulated in the adult brain (Ekonomou et al., 2005; Kan et al., 2007; Pevny et al., 1998). Nevertheless, Sox1 expression has been described in the postnatal brain in some ventral brain regions (Kan et al., 2007) and in the sub granular zone (Venere et al., 2012). The only described non-neuronal expression of Sox1 is in lens fiber cells where it regulates the γ-cristallin genes during development (Nishiquchi et al., 1998). This model has only been used in a few studies (Arnold et al., 2008; Huang et al., 2014), yet the validity of this

model to delete genes in the adult brain has not yet been described. Here, we describe that the Sox1-Cre line has a slight effect on the weight of the adult brain, while no brain abnormalities were observed.

Results

Cre expression under the Sox1 promoter results in reduced brain weight

Even though the Cre-*loxP* system is widely used to generate tissue or cell specific knockout mouse lines, it is known that Cre expression can have a negative effect on the viability of cells in culture (Loonstra et al., 2001) and on embryonic development (Naiche and Papaioannou, 2007). In many Cre mouse strains, off-target effects have been investigated. However, the consequence of Cre expression under the Sox1 promoter (Takashima et al., 2007) has not yet been described. Cre expression under the Sox1 promoter should result in similar expression patterns as has been described for Sox1. During mouse development, Sox1 expression has been described to start as early as embryonic day 7.5 in the neuronal plate (Pevny et al., 1998). The Sox1 expression is present in the subsequent stages of brain development: the neuronal fold at E8.5 and the neuronal tube at E9.5 (Takashima et al., 2007), followed by expression in the forebrain and lens (Ekonomou et al., 2005; Nishiquchi et al., 1998) (Figure 1A).

We found that the Sox1-Cre mice have a significantly reduced brain weight at 1 and 3 months compared to WT littermates, of subsequently 7.9 and 4.0 percent ($p=0.044$, $p=0.011$, $n=6$, T-test; one-way) (Figure 1B and 1D). No effect of Cre on overall body weight was observed at these time points ($p=0.081$, $p=0.132$, $n=6$, T-test; one-way) (Figure 1C). Further investigation into the brain histology by Hematoxylin and Eosin staining showed no gross abnormalities in the different brain regions of the Sox1-Cre mice, like degeneration, apoptosis or necrosis. However, minimal diffuse cortical atrophy that was characterized by thinning of the grey and white matter, neuronal and glial cell nuclear condensation and minimal ventricular dilation was observed (Figure 2A). This could be observed as a reduction in the cortical surface area ($p=0.047$, $n=3$, T-test) (Figure 2D). Nuclei count of the cortex and dentate gyrus of the hippocampus showed no effect on cell number in these regions (Figure

2B and 2C). Though we found a small reduction in brain weight and cortical surface in the Sox1-Cre mice, no clear pathological abnormalities could be found in the brains of these animals. Suggesting that except the slight reduction in overall size, no major brain defects are present in the Sox1-Cre animals.

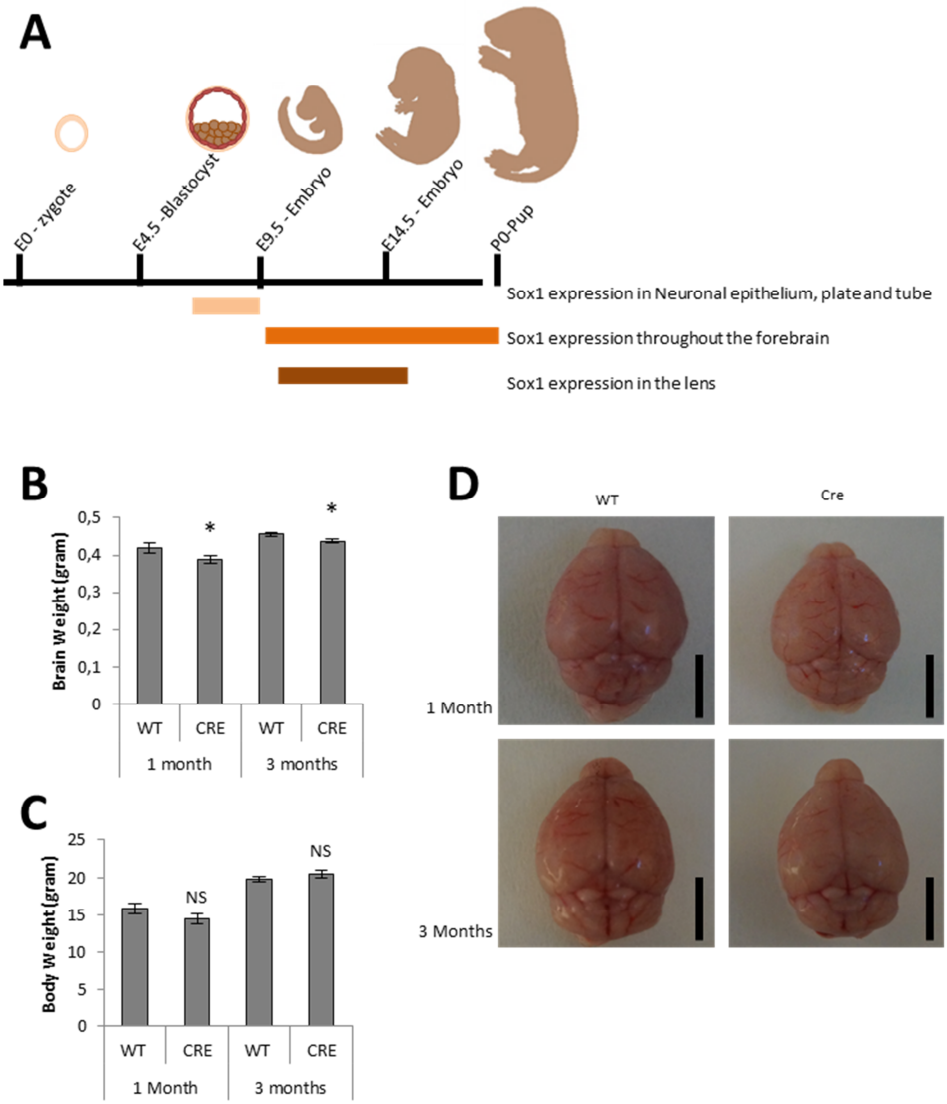

Figure 1. Sox1-Cre mice have slightly reduced brain weight. (A) Overview of Sox1 expression throughout development. **(B)** Brain weight of 1 and 3 month old WT and CRE female mice (n=6). (C) Body weight of 1 and 3 month old WT and CRE female mice (n=6). **(D)** Superior view of female WT and CRE brains at 1 and 3 months, scale bar is 5 mm.

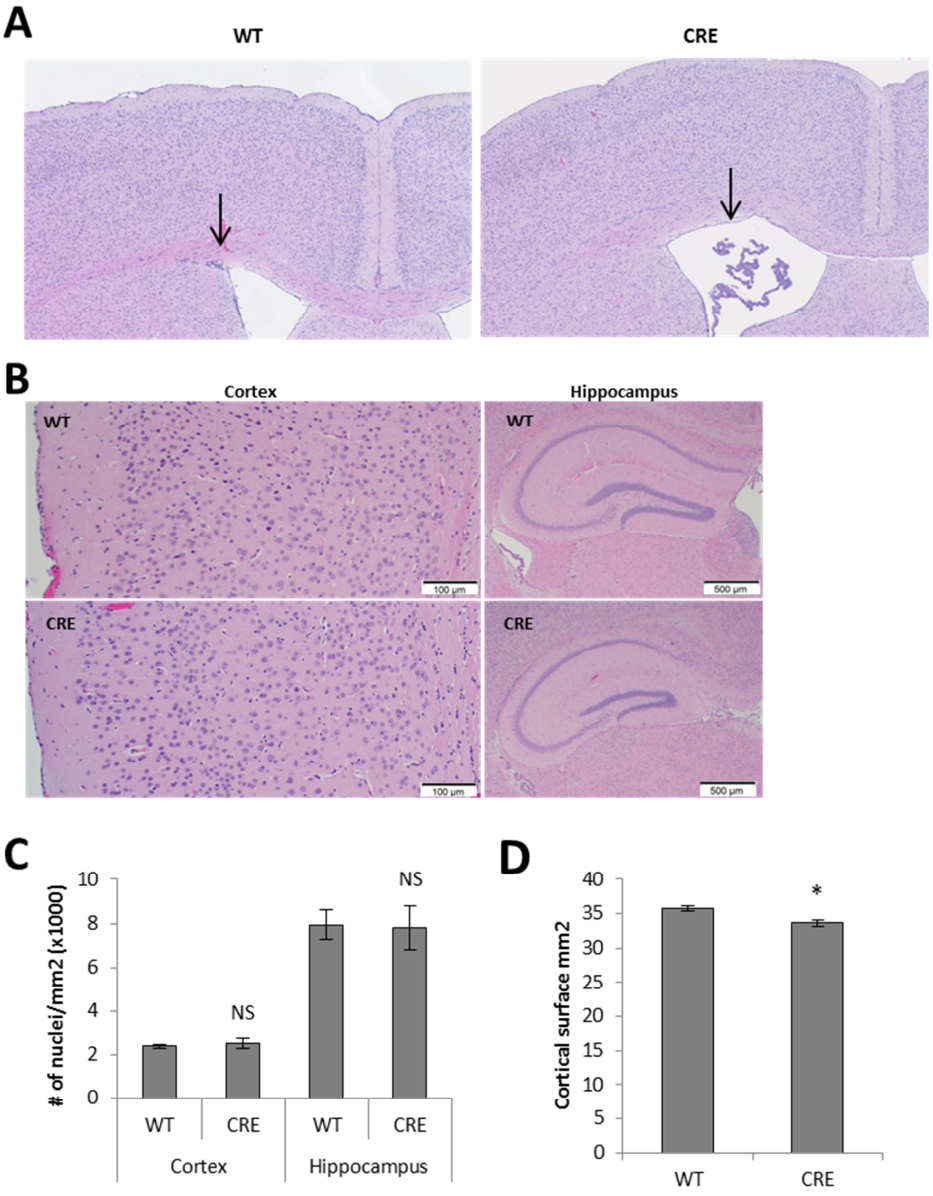

Figure 2. Sox1-Cre mice have slightly reduced cortical surface area but no overall brain abnormalities. (A) Representative photograph of the cortex of an 11 month old female WT and CRE mice, black arrows point out ventricle dilation. **(B)** Nuclei count of the cells in the cortex and dentate gyrus of the hippocampus in 3 month old WT and CRE female mice (n=3, mean ± SEM, t-test, NS). **(C)** Hematoxylin and Eosin staining of the hippocampus and cortex of 3 month old WT and CRE female mouse. **(D)** Cortical surface analyses (mm^2) in 11 month old WT and CRE female mice (n=3, mean ± SEM, t-test, *<0.05).

Discussion

Our data suggest a slight reduction in brain weight and cortical surface in the Sox1-Cre mouse model (Takashima et al., 2007), without any obvious pathological brain abnormalities. Two studies that use the Sox1-Cre mouse model to generate a brain specific gene deletion also described a reduction of brain weight, which they correspond to knockout of their gene of interest (Arnold et al., 2008; Huang et al., 2014). The first study investigates loss of the transcription factor Eomes/Tbr2 in early brain development on the embryonic subventricular zone (Arnold et al., 2008). They describe a reduced brain weight at postnatal day 0 and 60, but no effect on body weight, similar to what we observe. The effect they observe on cortical thickness we only observed at 3 months but not at 1 month (data not shown). However, because of the size difference of the brains it was difficult to make accurate measurements of the cortical thickness at similar locations, and due to the low number of animals it is difficult to interpret these results. The effect on cell number in the dentate gyrus of the hippocampus they describe we did not observe, nor did we observe an effect on cell number in the cortex (Figure 1F). Suggesting that the effect of Tbr2 on cell number of the dentate gyrus was due to Tbr2 loss and not due to Cre expression. Furthermore, a study using the Nestin-Cre mouse model to delete Tbr2 also observed an effect on the brain weight (Kahoud et al., 2014). For the Nestin-Cre alone only an effect on body weight has been described (Declercq et al., 2015; Giusti et al., 2014), however it might also have an effect on brain weight. To our knowledge the direct effect of Nestin-Cre on brain weight has not been described in the literature. The effect on brain weight in the Trb2 knockout mice for both the Nestin-Cre and the Sox1-Cre mice could also be an added effect of Cre, like we see for Serf2 (Chapter 5). The second study that uses the Sox1-Cre mouse model, investigates the loss of Cxcr4 on Purkinje cells of the cerebellum in the adult brain (Huang et al., 2014). Here a clear effect on brain size at postnatal day 9 was observed. The anatomical disruption of the cerebellum that is described is not observed in our study, showing that this effect is not due to Cre expression but to deletion of Cxcr4. Both studies did not investigate the effect of Cre expression by itself to exclude its effect. The toxic effect of Cre expression in mice and the need to add Cre-positive controls to mouse studies has been described over a decade ago (Naiche and Papaioannou, 2007), when reduced hematopoiesis and

increased apoptosis was described in embryonic tissue in three independent Cre lines.

Another explanation for the reduced brain weight could be the Sox1 heterozygosity in the Sox1-Cre line, because the Cre gene was inserted into the Sox1 open reading frame. Mice lacking the Sox1 gene show lens defects and suffer from spontaneous seizures between 4 and 6 weeks of age, that can be linked to defects in the ventral forebrain (Malas et al., 2003). Absence of major CNS defect might be a result of the highly homologous genes Sox2 and Sox3, that are also expressed throughout the development (Bowles et al., 2000). An effect on brain weight was not described for the Sox1 knockout mice. In this study we did not investigate the effect of Sox1 heterozygosity during brain development or in the adult brain.

Our study shows that the Sox1-Cre mouse model has a slightly reduced brain weight either due to Cre toxicity or due to heterozygosity of Sox1. However, no clear pathological abnormalities could be identified, nor an effect on apoptosis or proliferation, the effect of this Cre model on behavior has yet to be determined. Nevertheless, our study shows the importance to include the Sox1-Cre line as a control in future studies that use this model.

Experimental Procedures

Mouse breeding

Heterozygous Sox1-Cre mice (Takashima et al. 2007) were backcrossed at least 8 times to a C57BL/6J background and housed in individual ventilated cages on a 12-hour day/night cycle with ab libitum access to food. The body and brain weight for female mice was measured at 1 and 3 months. For genotyping, DNA from ear biopsies was purified using prepGEM® Tissue kit according to a protocol adapted from the manufacturer (ZYGEPTI0500, ZyGEM, VWR International BV, Amsterdam, the Netherlands) and subjected to PCR to amplify the Cre gene with the following primers Cre-F: AGCCTGTTTTGCACGTTCACC and Cre-R: GGTTTCCCGCAGAACCTGAA resulting in a 214 basepair product. Products were visualized on a 2% agarose gel. All experiments were approved by the ethical research committee for animal welfare at the RUG.

Brain processing and H&E staining

Brains were fixed in 4% formalin (Kinipath) for a minimum of 24 hours at room temperature. For the pathological analysis the brains were cut coronal and embedded in paraffin. 4 µm sections were cut using the microm HM 340E (Thermo Scientific), and incubated at 60°C for 15 minutes followed by deparaffinization and rehydration in xylene (2x), 100% alcohol (2x), 96% alcohol, 70% alcohol and water. Next, 4 minute incubation with hematoxylin, 10 minutes water and 1 minute eosin and 10 seconds water, followed by dehydration step of 2x 70% alcohol (2x), 96% alcohol (2x), 100%, alcohol (2x) and xylene (2x).

Nuclei Count

Hematoxylin and eosin stained coupes were scanned with the TissueFAXs microscope using 20X objective. Images were processed using Histoquest software, selecting either the cortex or the dentate gyrus for analyses.

Cortical surface measurement

Using ImageJ analyses representative pictures from the cerebral cortex at the coronal level of the corpus callosum/caudo putamen/basal forebrain were taken (equivalent to coordinates = Bregma:0.38 mm/Interaural: 4.18 mm) and the cortical surface was measured using the following macro:

```
run("16-bit");
```

```
setAutoThreshold("Default");

//run("Threshold...");

setThreshold(31, 230);

makeRectangle(54, 126, 1736, 1016);

run("Measure");
```

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